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Reversible and Preventable by Exogenous Decorin

PRINCIPAL INVESTIGATOR: Paul Okunieff, M.D.
Ivan Ding, M.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14627

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Cutaneous fibrosis after breast cancer radiotherapy is unavoidable, progressive, and irreversible. Currently there is no satisfactory, preventive therapy to ameliorate this process. Overexpression of fibrogenic cytokine TGFβ has been associated with soft tissue fibrosis, and reduction of its production or activation can ameliorate radiation induced normal tissue toxicity. Decorin is a small, leucine-rich proteoglycan, and is thought to be a natural inhibitor of TGFβ. In this study, we investigated whether decorin can reduce or prevent radiation induced acute and chronic damage to cutaneous tissue in mouse model. We showed: 1) Inflammatory changes of the papillary dermis in several mouse strains occurred within two weeks of irradiation. Radiation-induced cutaneous toxicity was associated with induction of inflammatory cytokines (IL-1 and TGFβ) and chemokine (MCP-1); 2) Mouse strain dependency of radiation induced cutaneous toxicity was associated with TGFβ blood levels; 3) Intramuscular injection of an TGFβ1-expressing adenovirus induced pathological alterations in skin a week after injection that were similar to those caused by radiation. In addition, overexpression of TGFβ in cutaneous tissue facilitated radiation induced soft tissue fibrosis; 4) Decorin not only decreased TGFβ1-mediated PAI-1 promoter activity in vitro, it also reduced bleomycin toxicity in lung in vivo. 5) Pre-radiation injection of a decorin-expressing adenovirus did not significantly reduce radiation-induced acute skin toxicity, but injection 40 days after radiation marginally ameliorated late tissue fibrosis. Our results suggest that elevation of TGFβ plays a critical role in cutaneous radiation damage, and decorin can reduce TGFβ-mediated fibrogenic process.				
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Table of Contents

Cover

SF 298 ----- 1

Introduction----- 3

Body ----- 3

Key Research Accomplishments----- 5

Reportable Outcomes ----- N/A

Conclusions ----- 5

References ----- 5

Appendices ----- 6

List of Personnel ----- 14

Fibrosis of the Breast Skin after Irradiation is Reversible and Preventable by Exogenous Decorin

INTRODUCTION

Cutaneous fibrosis after breast cancer radiotherapy is considered unavoidable, progressive and an irreversible side effect of treatment. At present there are no satisfactory therapies, no assays to predict which patients will develop severe reactions, and no preventive strategies to ameliorate early inflammatory and late (fibrosis) cutaneous toxicity. More importantly, the fibrotic process can result in breast and arm edema, weakness, and pain. At least 50% of women have some complications from this radiation-induced normal tissue damage to the arm, lung, or breast, and this affects quality of life in at least 20% of these patients. Currently, the mechanism of radiation-induced cutaneous fibrosis is unknown. Radiation fibrosis is a complex process involving unknown cellular, genetic, and microenvironment interactions. Cell survival does not correlate with fibrosis sensitivity, and studies using animal models are needed. The hypothesis of this investigation is that fibrosis of the skin after irradiation for breast cancer radiotherapy is reversible and preventable by control of the Decorin/TGF β 1 ratio in local tissues. Our preliminary data showed that inflammatory changes in the papillary dermis of several strains of mice occur within 2 weeks of irradiation. The fibrosis spreads from superficial of dermis to involve the full dermis. We observed identical pathological alterations seen in skin a week after intramuscular injection of an adenovirus overexpressing active TGF β 1 (1). Similarly, we have seen histological lung inflammatory reactions that are indiscernible from radiation fibrosis of the lung after intrapulmonary instillation of TGF β 1 virus (2, 3). Decorin is a small proteoglycan that binds to the TGF β core protein and inhibits multiple TGF β isoforms. Decorin has been shown to alleviate TGF β 1 induced fibrosis of the lung and kidney, whether given as recombinant protein or as in-vivo gene therapy (4-8). Indirect evidence also attributes low decorin to gliosis in the brain, and to myocardial and hepatic fibrosis. These processes all histologically resemble radiation fibrovascular toxicity in cutaneous tissues. Therefore, we propose that late radiation fibrosis is due to slowly dividing cells eventually dying and then being passively replaced by fibrosis (over growing fibroblast and proliferation) mediated by disturbance between TGF β 1 and decorin. In this proposal we will investigate if radiation fibrosis is an active process, requiring continuous maintenance by TGF β 1 production and low decorin in murine radiation-induced cutaneous fibrosis models. If our hypothesis proves correct, we will have a predictive assay (TGF β 1/decorin ratio), a preventive strategy (administration of decorin in early stage), and a therapeutic mean (application of decorin in late stage) for late radiation fibrosis (LRF). The clinical implications are great for breast cancer, as well as many other cancers that require radiation therapy.

PRELIMINARY RESULTS

1. Radiation-induced skin TGF β 1 mRNA in C3H/HeN mice correspond to the level of early radiation dermatitis (ERD).

C3H/HeN mice were given 50 Gy irradiation into right hind leg, and the left leg was used as non-radiation control. Radiation induced cytokine and chemokine mRNA expression was determined by RNase protection assay shown in Figure 1a. Even among animals of the same strain, there is inter-animal variability of ERD and cytokine mRNA expression levels. If the ERD skin score seen at day 15 following irradiation is plotted along with mRNA levels of cutaneous IL-1 β or MCP-1, there is a significant correlation (Fig 1b). This result supports an interaction between IL-1 β and MCP-1 with ERD. The data also further suggests that the severity of ERD is modulated by the level of these two cytokines. TGF β 1 appears to primarily alter the severity of LRF (this will be shown later) and thus showed little correlation with ERD, although elevated TGF β mRNA was observed 15 day after radiation. As shown above we have also performed experiments at radiation doses ranging from 10 to 60 Gy. The latency to ERD is only minimally modified by radiation dose. In contrast, the severity of ERD is affected by dose. Late effects have identical pathology independent of radiation dose. In the case of late effects however, there is little late fibrosis for single doses under 20 Gy in any mouse strain, and the latency is over three month. At 30 Gy the latent period to development of maximal LRF requires 0.5 to 1.0 year depending on strain. At higher doses (40 to 50 Gy), the LRF reaction becomes maximum in 2 months in C57Bl/6 mice, and 3-4 months in C3H/HeN mice (Fig 2). Further increases in dose do not further shorten latency.

2. Levels of TGF β 1 mRNA in the skin after radiation, and even circulating TGF β 1 protein levels of C3H/HeN and C57Bl/6 mice correspond to the level of LRF.

Radiation induced cutaneous fibrosis increased with time in both C3H/HeN and C57Bl/6 mice (Figure 2). Three months after 45 Gy radiation in right hind leg, over 80% of C57Bl/6 mice developed severe delay radiation fibrosis (DRF) scores whereas most of the C3H/HeN mice had only a moderate DRF. Thus, in design of late effect studies it is important to choose a radiation dose and a time of assay. It is also valuable to measure the time course of DRF score, since the more fibrotic strains have a steeper rate of fibrosis development, such as C57Bl6. In general LRF increases in time and with

dose. These observations make experimental design simple, in that late effects can be determined with 2-3 months latency if one uses doses of 30 to 40 Gy in C57Bl/6 mice. C3H/HeN mice can be used as confirmation, with similar doses and latency periods of about 3-4 months. Skin tissue TGF β 1 mRNA expression in the late stage of radiation are shown in Figure 3. C57Bl/6 mice were given 20 Gy to their hind leg and followed for up to 2.5 years. TGF β 1 mRNA from skin and muscle were measured by RNase protection assay. There was substantial animal variation in TGF β 1 expression level among individual animals. Control animals consistently had low TGF β 1 expression. At 20 Gy, the degree of fibrosis varies with time and between animals. Consistent with this, the worst skin scores corresponded to the animals with the highest elevation of TGF β 1. "C" signifies the control non-irradiated age-matched animals. Other lanes are individual treated animals

Individual mouse stains have naturally different levels of circulating TGF β 1. All four mice strains had blood collection in EDTA anti-coagulation with platelet poor centrifugation. Blood TGF β 1 was measured by ELISA, shown in Figure 4. Mouse strains included C3H/HeN, BALB/c, and TGF β 1[+/+] and [+/-] littermates at various ages ranging from 1 to 6 months. TGF β 1 levels were measured by quantitative ELISA (5-10 mice per point, mean \pm 1 SE). C3H/HeN and the TGF β 1[+/-] animals had low circulating TGF β 1 levels, while the C57Bl/6[+/+] had the highest levels of circulating cytokines. TGF β 1[+/+] and [+/-] were inbred back into C57Bl/6. The degree of DRF at 3 months corresponds well with the circulating endogenous level of TGF β 1. Specifically, C3H/HeN and TGF β 1[+/-] mice have the lowest fibrosis and blood TGF β 1 levels. Circulating TGF β 1 levels are intermediate in the BALB/c mice, who are also intermediate DRF strains, and fibrosis is most severe in the TGF β 1 wild type C57Bl/6 background mice.

3. Intramuscular injection of TGF β 1/Ad5 provide very high protein expression levels for 3 to 7 days, and vector controls have very little inflammation of the skin.

Although overexpression of cytokine or chemokine in the local tissues can be obtained by direct injection of cytokine/chemokine adenovirus to tissue, adenoviral vectors have shown inflammatory responses in some experimental models and have different durations of expression and levels of expression in different tissue types. We studied the effects of overexpression of TGF β 1/Ad5 inflammatory response on our radiation induced murine soft tissue fibrosis models. The Ad/GFP vector control produces little if any detectable inflammatory response during day 3 to day 7, as shown in Figure 5. GFP fluorescing documented maximal expression at day 3 with good expression at day 7 after GFP/Ad intramuscular injection. However, GFP was low at 14 days (Figure 5). TGF β 1 produced a remarkable inflammatory reaction. The duration of expression is convenient to our experimental design for which expression is ideally needed for 3 to 7 days. We tested the specificity of adenoviral vectors to produce the cytokine of interest. Our results support the utility of this model system. Adenoviral vector alone, though clearly immunogenic in some animal systems, does not greatly affect the levels of any of the cytokines, such as IL-1b and MCP-1, that are being targeted in cutaneous tissues (Figure 6a). As previously shown, the adenoviral vector does not cause substantial inflammatory cell infiltration, nor does it increase MCP-1. The Ad/TGF β 1 in contrast does cause mononuclear cell infiltration. Overexpression of TGF β -enhanced radiation acute soft tissue toxicity was also observed in the early stage of normal tissue damage (day 17). As shown in Figure 6b, mice treated with 50 Gy alone had less skin toxicity compared with radiation plus Ad/TGF β 1 adenovirus injection.

4. Decorin/Ad5 vectors provide very high protein expression levels in vivo, and prevent bleomycin-induced pulmonary fibrosis in mice.

Using Ad/Decorin-transfected mink lung epithelial cells (MLEC) cells with a stable transfected human plasminogen activator inhibitor-1 (PAI-1) promoter, we demonstrated that decorin dose-dependently reduced active TGF β (1:4 from condition medium) mediated PAI-1 promoter transcription, shown in Figure 7a. A luciferase reporter assay is used in this study. In studies of bleomycin-induced lung fibrosis, we also showed that there was a huge decrease in collagen deposition in Ad/Decorin-treated animals (Figure 7b). Bleomycin toxicity in the lung has many similarities with radiation fibrosis of the lung. We have also found that radiation acute toxicity in the lung is similar to radiation acute cutaneous tissue toxicity in other experiments. Specifically, TGF β 1 appears to be a critical modulator of early and late effects. Administration of Decorin/Ad5 significantly reduced bleomycin induced lung toxicity.

5. Decorin did not radioprotect early and late cutaneous toxicity from a single high dose of radiation (50 Gy).

As we proposed, decorin might reduce or prevent radiation induced soft tissue damage if administration of decorin is appropriate in terms of dose or time. C57BL/6 mice were given one dose of decorin/Ad at day -2 (early) and day 40 (later), and then mice were irradiated to hind leg at day 0. Acute skin scores (day 16 to day 19) and chronic skin score (90 day post radiation) were determined (Figure 8). Decorin did not radioprotect cutaneous tissues if administration was given during early stage of radiation. Late application of decorin (40 days after radiation) caused marginally significant radioprotection of cutaneous tissues compared with vector controls. Taken together these data support that decorin-mediated reduction of TGF β may occur in an organ-dependent manner.

KEY RESEARCH ACCOMPLISHMENTS

1. There is a clear effect on the cutaneous soft tissues by radiation on the levels of TGF β 1, IL-1 β and MCP-1mRNA expression, as well as a clear effect on macrophage infiltration.
2. There is a strong correlation between IL-1 β and MCP-1 with ERD, and there is a strong association between TGF β 1 and DRF.
3. Differences in DRF correspond with levels of naturally circulating TGF β 1. Expression of TGF β 1 differs between mouse strains in accordance with their sensitivity to DRF.
4. Adenoviral vectors cause little if any observable cutaneous inflammation on their own, and among the cytokines of interest, adenoviral vectors expressing a specific cytokine specifically increase the mRNA and protein at biologically active levels.
5. Adenoviral decorin transfection can reduce the transcription regulation of activated TGF β 1 and prevent bleomycin induced pulmonary fibrosis in mice.
6. Adenoviral vectors provide a brief overexpression of cytokine lasting at least 3 to 7 days, and often 10 days. They thus can conveniently be used to block the known periods of radiation induced over-expression of cytokines.
7. Overexpression of decorin at the early stage of radiation did not protect radiation cutaneous toxicity, and possible radioprotective effects were observed when decorin were given at late stage of radiation.

CONCLUSION

Although we have demonstrated that causal relationship exists between overexpression of active TGF β 1 and acute inflammation in cutaneous tissues after intramuscular injection of Ad/TGF β 1, we did not prove the existence of modulatory effect of decorin on the TGF β 1 activity in vivo; we only showed that administration of decorin/Ad at 40 days after radiation had a slightly radioprotective effect on chronic skin toxicity after a single 50 Gy leg radiation. Because administration of decorin in the early stage of radiation did not offer any radioprotective effects to soft tissue, it may suggest that elevation of TGF β likely is a late event of radiation, and elimination of TGF β in the late stage may benefit cutaneous radioprotection.

REFERENCE

1. Ding, I., Liu, W. M., Hu, D. P., William, J., Okunieff, P., and Sime, P. J. Intramuscular injection of single dose of adenovirus expressing active TGF β 1 induces acute inflammation and facilitates radiation cutaneous fibrosis, 2nd Int Conference on Translational Research and Pre-Clinical Strategies in Radiation Oncology. (submitted), 2003.
2. Gauldie, J., Sime, P. J., Axing, Z. and Tremble, G. M. Transforming growth factor-beta gene transfer to the lung induces myofibroblast presence and pulmonary fibrosis, *Curr Top Pathol.* 93: 35-45., 1999.
3. Liu, J. Y., Sime, P. J., Wu, T., Warshamana, G. S., Pociask, D., Tsai, S. Y., and Brody, A. R. Transforming growth factor-beta(1) overexpression in tumor necrosis factor-alpha receptor knockout mice induces fibroproliferative lung disease, *Am J Respir Cell Mol Biol.* 25: 3-7., 2001.
4. Schaefer, L., Macakova, K., Raslik, I., Micegova, M., Grone, H. J., Schonherr, E., Robenek, H., Echtermeyer, F. G., Grassel, S., Bruckner, P., Schaefer, R. M., Iozzo, R. V., and Kresse, H. Absence of decorin adversely influences tubulointerstitial fibrosis of the obstructed kidney by enhanced apoptosis and increased inflammatory reaction, *Am J Pathol.* 160: 1181-91., 2002.
5. Kolb, M., Margetts, P. J., Sime, P. J., and Gauldie, J. Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung, *Am J Physiol Lung Cell Mol Physiol.* 280: L1327-34., 2001.
6. Kolb, M., Margetts, P. J., Galt, T., Sime, P. J., Xing, Z., Schmidt, M., and Gauldie, J. Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin, *Am J Respir Crit Care Med.* 163: 770-7., 2001.
7. Giri, S. N., Hyde, D. M., Braun, R. K., Gaarde, W., Harper, J. R., and Pierschbacher, M. D. Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis, *Biochem Pharmacol.* 54: 1205-16., 1997.
8. Isaka, Y., Brees, D. K., Ikegaya, K., Kaneda, Y., Imai, E., Noble, N. A., and Border, W. A. Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney, *Nat Med.* 2: 418-23., 1996.
9. Hitt, M. M. and Gauldie, J. Gene vectors for cytokine expression in vivo, *Curr Pharm Des.* 6: 613-32., 2000.
10. Gauldie, J., Kolb, M., and Sime, P. J. A new direction in the pathogenesis of idiopathic pulmonary fibrosis?, *Respir Res.* 3: 1., 2002.

Correlation of Cytokine mRNA Expression with Acute Skin Score

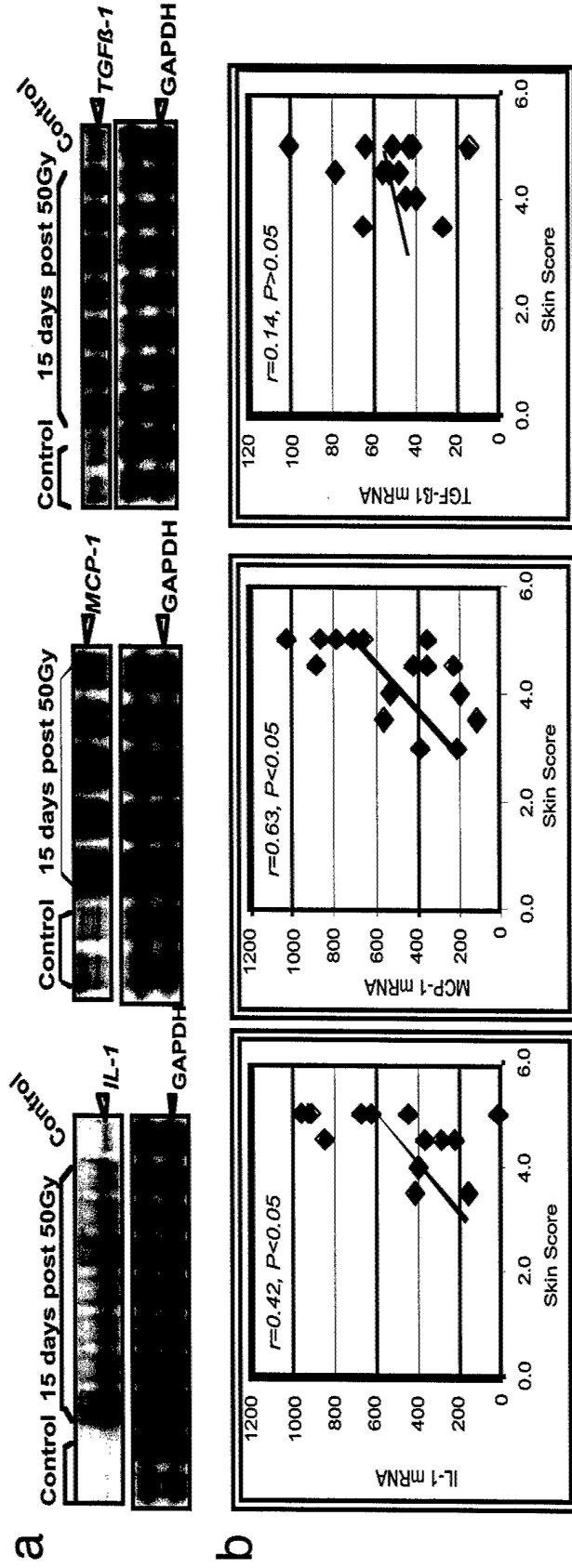


Figure 1. (a) Radiation induced cytokine and chemokine mRNA expression as determined by RNase protection assay. (b) Correlation of early radiation dermatitis with mRNA levels at day 15 following irradiation.

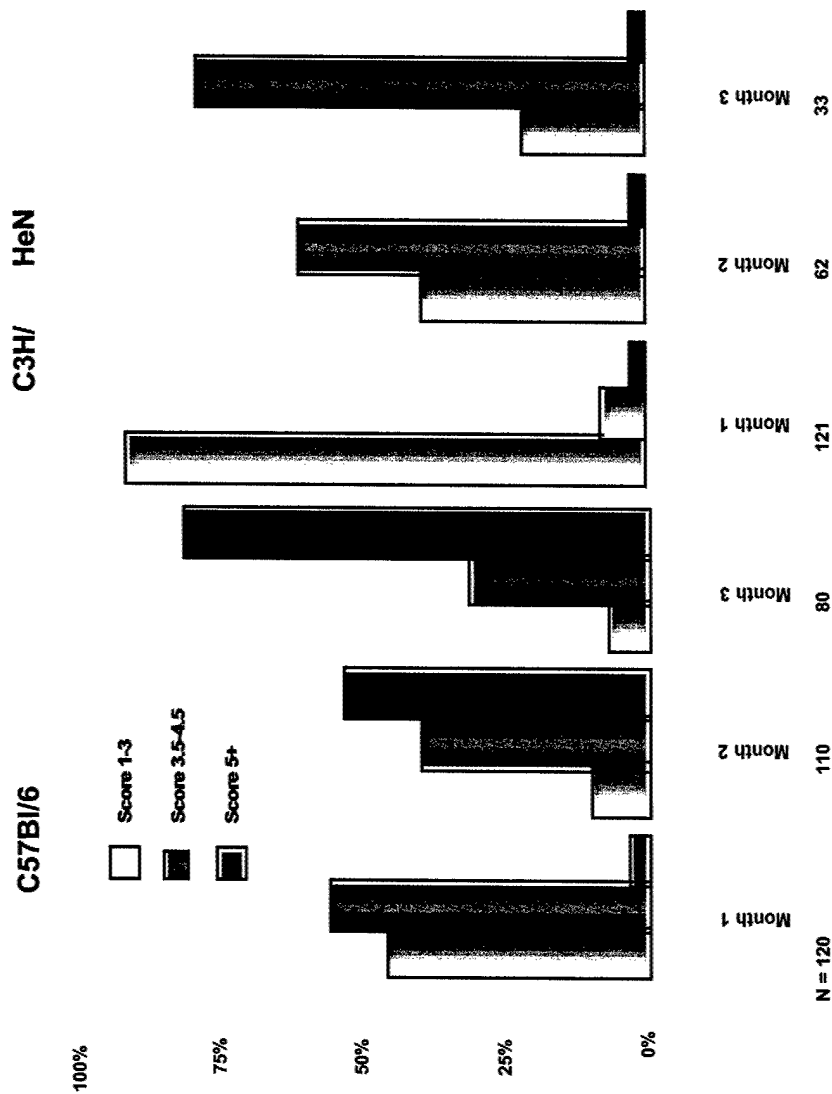


Figure 2. Extremity Fibrosis Score at Various Times Following Irradiation

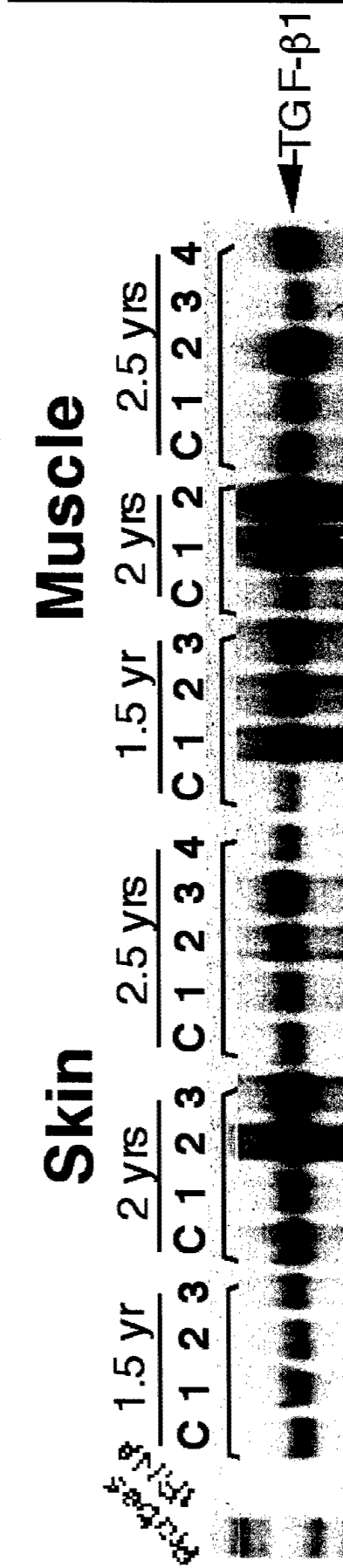


Figure 3. Skin tissue TGFβ1 mRNA expression in the late stage of radiation

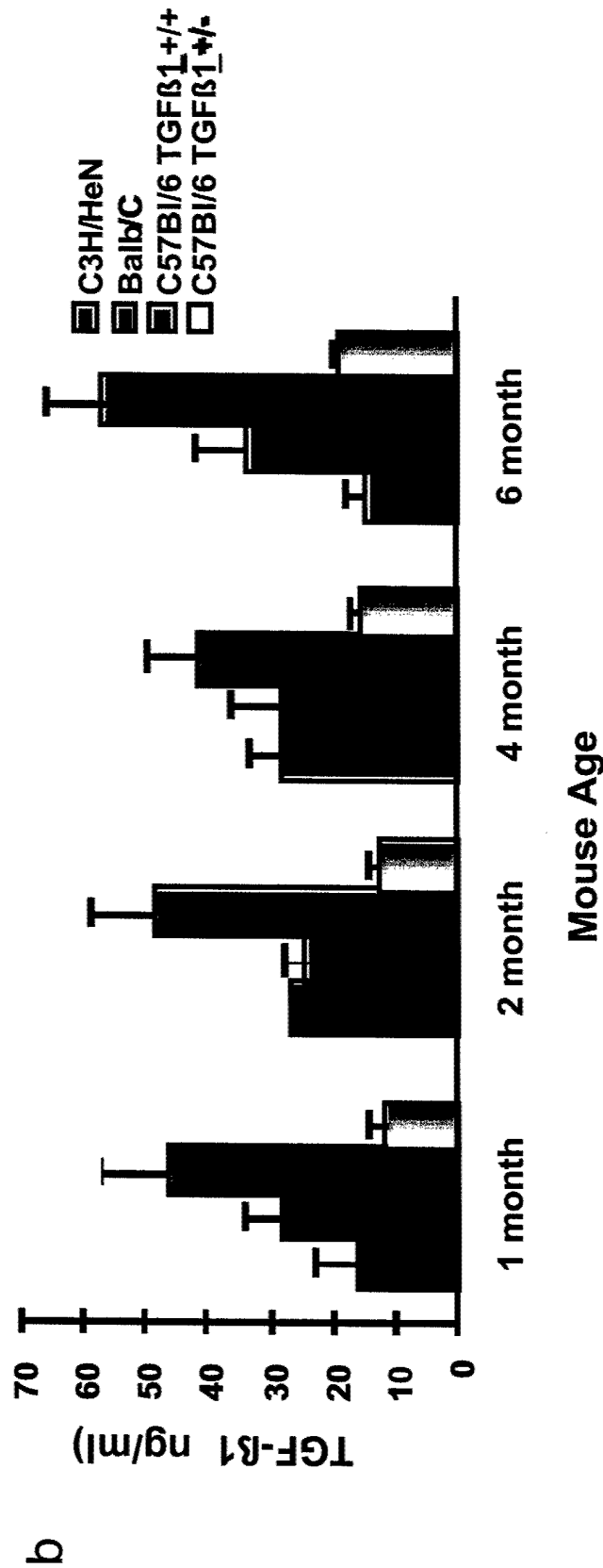
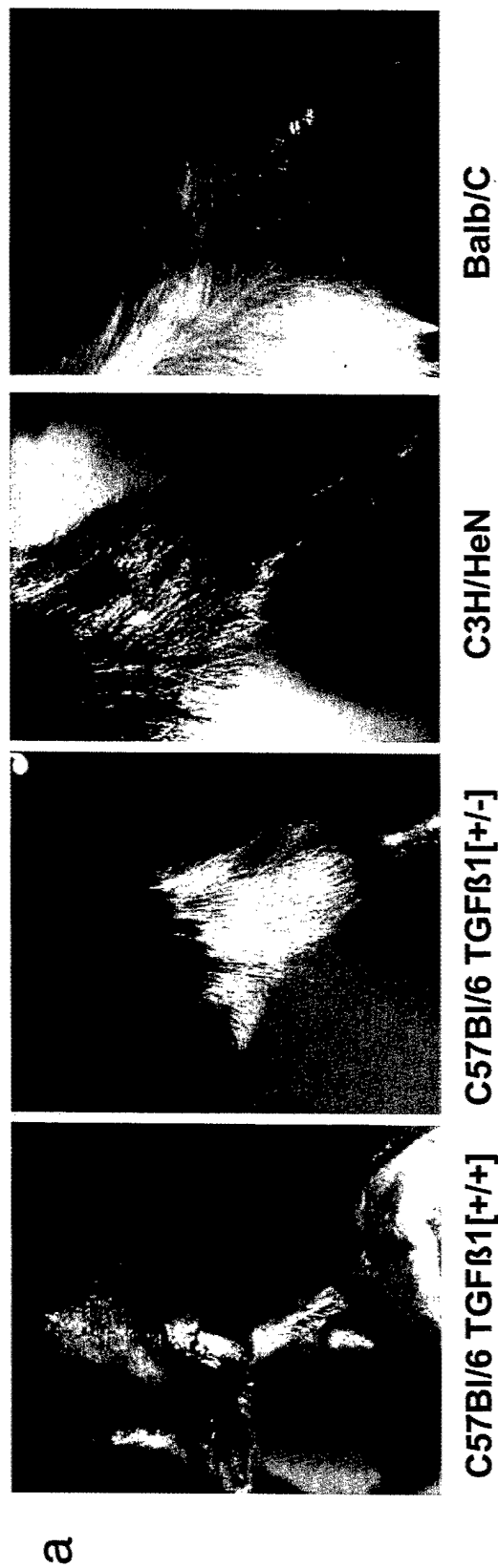
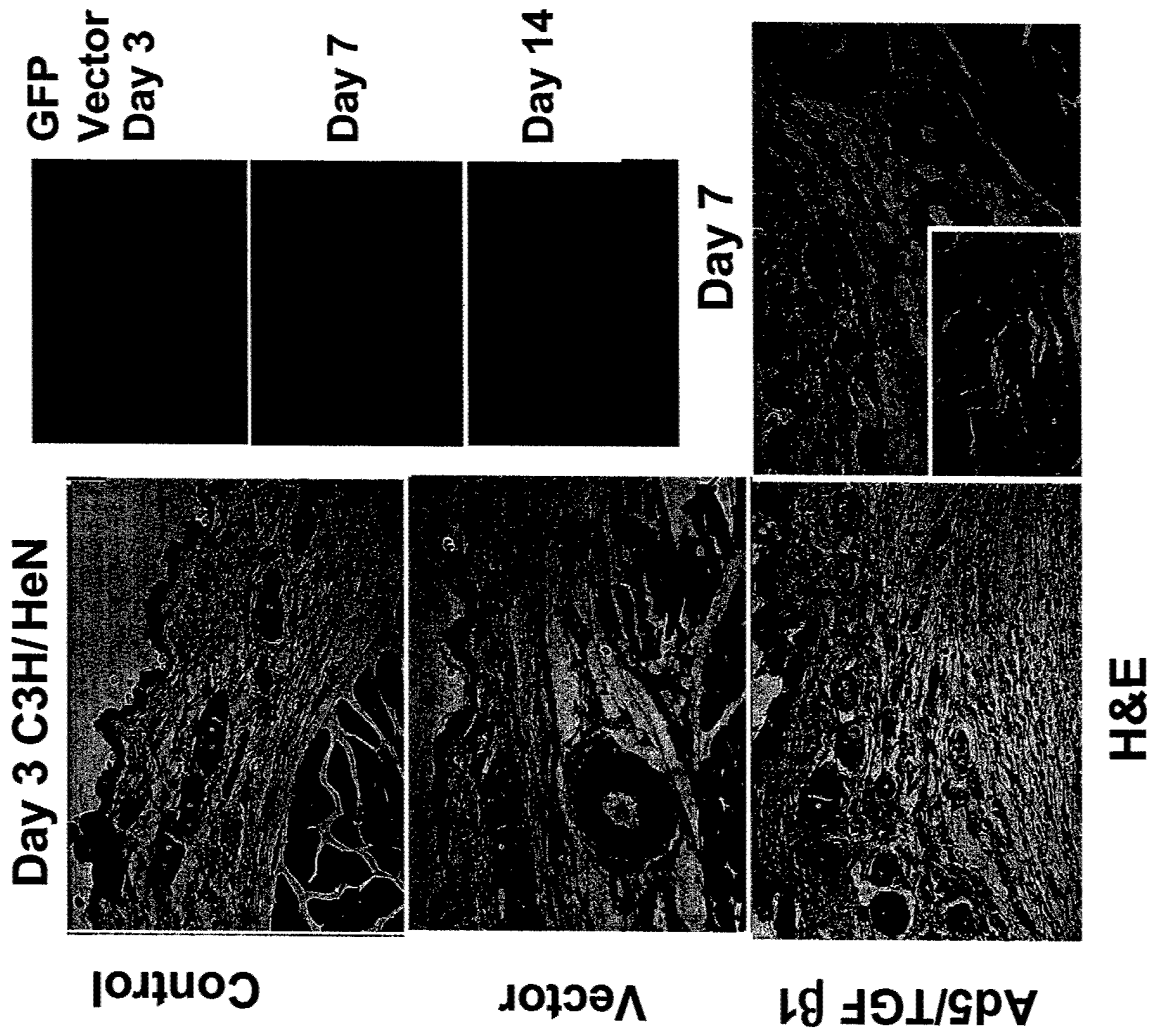
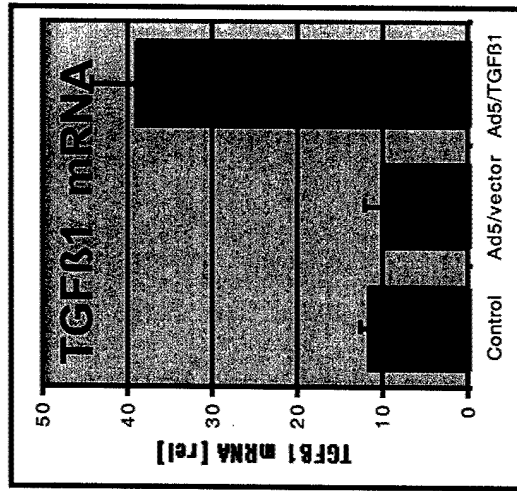
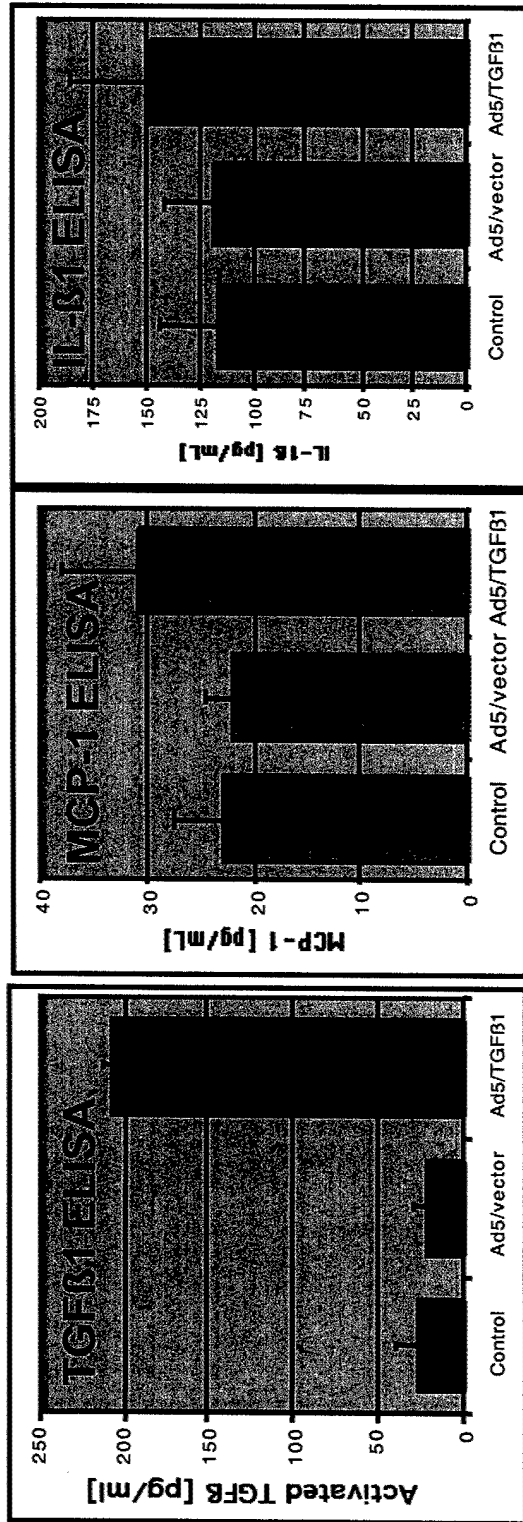


Figure 4. (a) Delay radiation fibrosis at 3 months post irradiation is shown for the four mice strains.. (b) Blood TGFβ1 in the four mice strains as measured by ELISA

- Figure 5
- Vector alone does not cause significant inflammation (see H&E)
 - Expression of vector is confirmed using GFP at 7 days
 - Ad5/TGFB1 induces inflammation beginning at 3 days and lasting for 7-10 days. The reaction includes thickened dermis and the inflammation also involves the skeletal muscle
 - These observations mimic some of the effects seen after irradiation, and include macrophage infiltration (immunohistochemistry not shown)





Cytokine mRNA expression using the AD5/TGFB β 1 compared to the AD5/vector and with respect to control untreated limb tissues

AD5/TGFB β 1 appear to be specific for TGFβ1.

There appears to be no increase in MCP-1 or TGFβ1 induced by the Ad/TGFB β 1.

- The vector alone has very little effect on IL-1 β or MCP-1 levels.

All values are mean \pm 1 SE

Figure 6a. Skin cytokine levels at 3 days

14 Days

90 Days



Ad/TGFβ1



Ad/DL



Figure 6b. Comparison of skin toxicity of mice treated with radiation alone and those treated radiation plus Ad/TGFβ1 adenovirus injection.

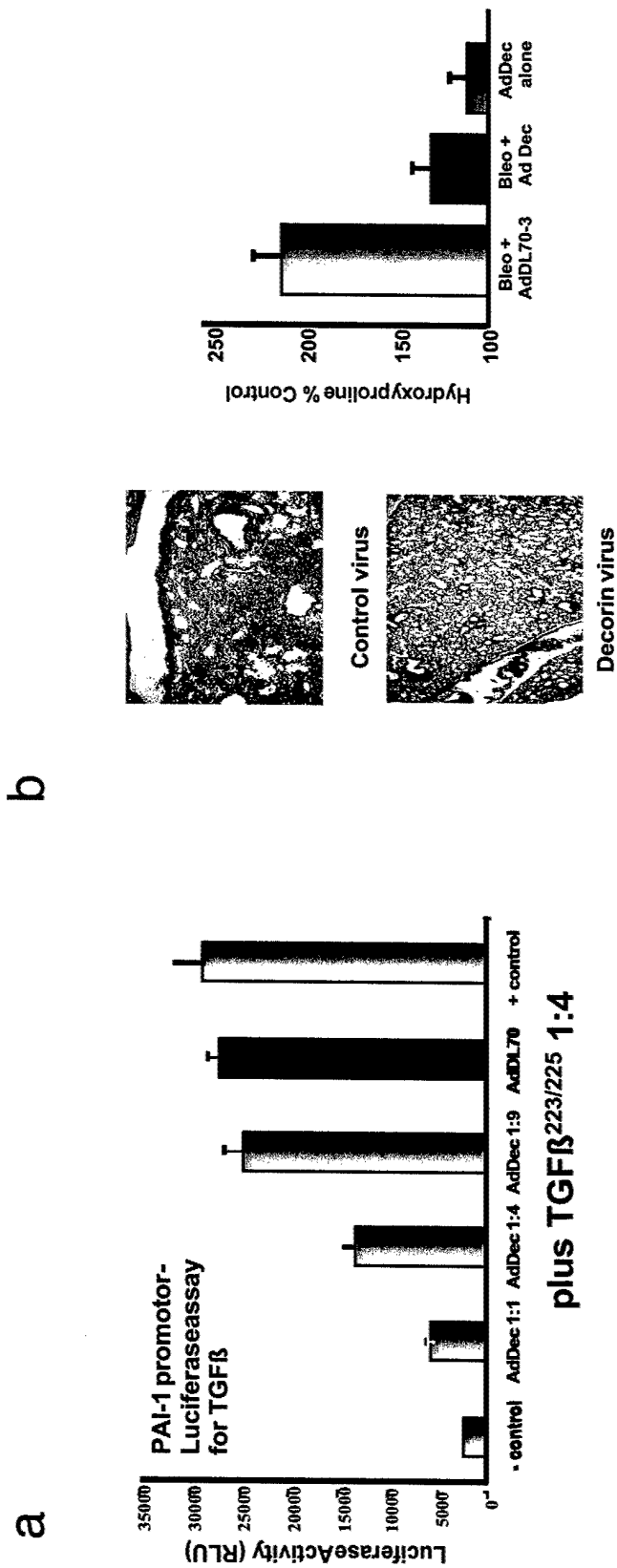


Figure 7. (a) Inhibition of TGF β activity by adenovirus decorin *in vitro*. (b) Inhibition of bleomycin-induced lung fibrosis by adenovector-mediated decorin over-expression

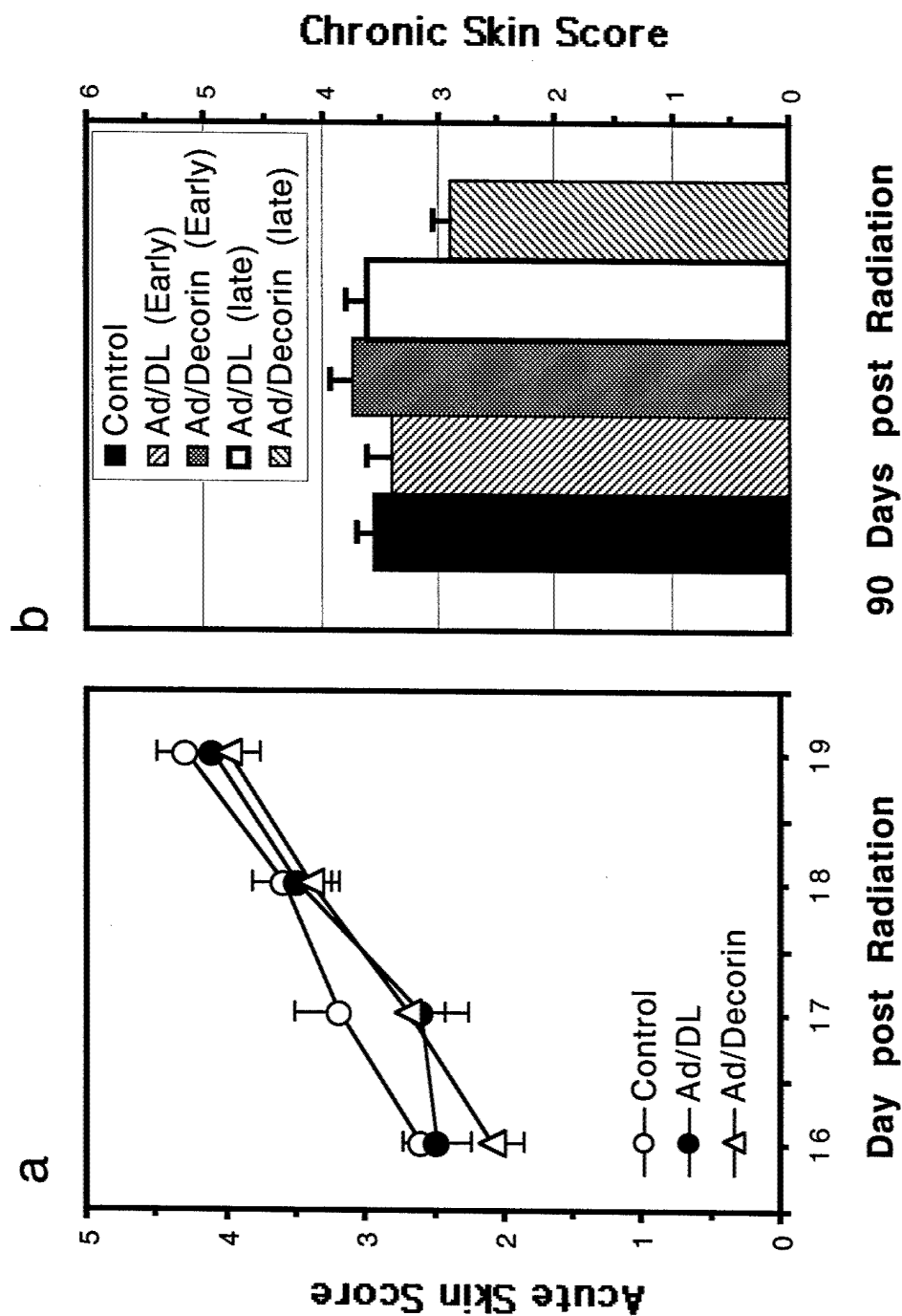


Figure 8. (a) Acute skin scores after receiving pre-irradiation decorin/Ad and (b) chronic skin scores 90 days post-irradiation after receiving early decorin/Ad (pre-irradiation) and late decorin/AD (40 days post-irradiation)

List of personnel receiving pay from this research effort

1. Chin-Rang Yang